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IONIC CHANNELS AS NATURAL NANODEVICES

Rush Presbyterian-St. Luke's Medical Center

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13. ABSTRACT ***(Maximum 200 Words)***

The goal of the project was to make ion channels into practical devices that can be controlled for medical and technological use. Ion channels are proteins embedded in the lipid membrane of biological cells. Because of their structural characteristics, ion channels are responsible for regulating the flux of ionic charge across the cellular membrane. This particular effort pursued the integration of natural ion channels and artificial microelectronic components in hybrid bio-devices that couple some of the best aspects of the two worlds. Once the control of channels is understood, the opportunity will exist to produce a technology as important for ions as transistors are for electrons. The focus of this effort evolved from the development of tools, to the use of those tools to make novel interfaces to ionic channels. The overall effort involved a combination of modeling, simulation experimentation, and fabrication of a demonstration vehicle for a concept of embedding channels in a membrane on silicon. Early activity in this project expanded the theoretical understand of ion channels. The resulting demonstration vehicle provides insight into the performance of ion channels, bringing together important building blocks for a fully integrated biosensor with on-chip sensing and signal processing.

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1.0 – Introduction

The goal of the project was to make ion channels into practical devices that can be controlled for medical and technological use. The intent of the work was to exploit the power of the engineering approach to develop devices by using models and experiments to design devices instead of trial and error experiments. In the engineering tradition great effort is spent constructing, simulating and testing models, because once a model is established that is useful in a reasonable domain of conditions, design is relatively easy and efficient. Once the control of channels is understood, the opportunity will exist to produce a technology as important for ions as transistors are for electrons. The focus of this particular effort, through several redirections from DARPA, evolved from the development of tools, to the use of those tools to make novel interfaces to ionic channels. The overall effort involved a combination of modeling, simulation, experimentation, and fabrication of a demonstration vehicle for a concept of embedding channels in a membrane on silicon. The resulting demonstration vehicle provided insight into performance of ion channels. It brought together important building blocks for a fully integrated biosensor with on-chip sensing and signal processing.

1.1 - Scientific Basis of Rationale

Ion-channels are proteins embedded in the lipid membrane of biological cells. Because of their structural characteristics, ion channels are responsible for regulating the flux of ionic charge across the cellular membrane. For instance, the generation and transmission of potentials in nerves and muscles, as well as the release of hormone from endocrine cells, are believed to be mechanisms governed by ion transport through these protein “gates”. Furthermore, from an engineering viewpoint, ion channels will be a key component of a new generation of biosensors that integrate the selectivity and extreme sensitivity of ion channels with the processing capabilities of modern microelectronics.

This work pursues the integration of natural ion channels and artificial microelectronic components in hybrid bio-devices that couple some of the best aspects of the two worlds. Indeed, ion channels are a prime candidate for biosensors because their extreme sensibility, sensitivity, and large signal-to-noise response. However, the use of biomolecules as practical sensors requires robust and reliable performance. The properties of ion channels, in particular, can be exploited in artificial devices only if their natural environment is adequately reproduced, meaning that ion-channels can be profitably used when embedded in cell membranes that provide the correct electrical environment.

Ion channels operate by using atomic events to control (i.e., ‘gate’) flows between macroscopic reservoirs of ions, that act as batteries. The macroscopic reservoirs maintain (reasonably) constant concentration and electrical potential, and thus constant electrochemical potential. If the channel is small enough, a gate that responds to atomic events can control the extremely small flux through the channel. Such control capability

can be used in man-made devices only if the resulting currents are reproducible and large compared to confounding background currents (flowing in parallel to the channel), and confounding potentials (in series with the channel).

Biological systems ensure the reproducibility of channel currents by going to exquisite lengths to maintain the proper electrical and chemical environment. Confounding parallel currents are kept tiny (< 0.1 pA) and the electrical potential across and in series with channels is maintained constant and noise free (i.e., noise less than 50 μV including 'dc' components of drift). In this way, the tiny currents through channels are maintained at strictly reproducible values uncontaminated by (variable) artifacts from systems in series or parallel.

The consequences of an uncontrolled potential across the channel are particularly serious, and so variations in parallel currents (that can change this potential profoundly albeit indirectly) and variation in series potential must be reduced to essentially zero. If the potential changes 50 mV or thereabouts, for a second or so, most channels go into inactive states, from which they can be reactivated only with difficulty, if at all. If the potential varies much more than that for much longer than that, most channels enter a profound inactive state and some "die", i.e., presumably denature. If the potential reaches 200 mV or so, even for ~1 msec, the channel and membrane develop irreversible leaks and can no longer be returned to their useful native state. This sensitivity to voltage is not unexpected given the small size of the channel protein and the consequent enormous electric fields.

Channels in biological systems are embedded in high resistance lipid membranes that reduce to negligible values the current flowing through parasitic conduction paths in parallel to the channels. The membranes that surround channels are remarkably impermeable to ions, although they are little more than soap films. The conductance of lipid membranes is typically measured in GΩ even though they are ~2 nm thick and are embedded in highly conductive ionic solutions! It should be noted that, in comparison, many solid-state insulators of our technology have resistances of ~100 MΩ distributed on similar length. Biologic systems also keep the potential in series with channels small and constant as a natural consequence of their ionic environment. These homeostatic systems are of great importance to the viability of cells. A substantial fraction of the physiology of cells (and organisms) is devoted to maintaining a constant environment, as reference to any textbook of physiology or cell biology will show, and the proximal cause of death is usually the failure to maintain that environment. Thus, physicians often study and know the physiological processes of homeostasis at least as well as physiologists and bioengineers.

In summary, the central issue in making a device out of ion channels is the creation of an environment that allows channel currents to be reproducibly large compared to parasitic background currents flowing in parallel with the channel, and the elimination of confounding potentials in series with the channel.

1.2 - Interdisciplinary Approach

The approach is necessarily interdisciplinary and inter-institutional because making ion channels into devices requires understanding:

- How to build a tiny experimental chamber that can be replicated to make arrays of addressable devices and can record stable currents free of artifact through individual ion channels.
- The electronics in recording picoamp currents from gigohm resistance devices in the presence of stray capacitances of picofarads.
- Experimental difficulties in handling lipid bilayers, proteins, and the electrochemical cells used to record from proteins in bilayers.
- The biology and technology of proteins that form them. Both biochemical and biophysical approaches are absolutely necessary, using the highly developed armamentarium of molecular biology and electro-physiology. As adept as the engineering community is, it cannot reinvent the armamentarium of tens of thousands of biologists working for 50 years by ignoring it. Indeed, if engineers do not identify the key physical, chemical, and technological limitations on manipulating and studying channels, engineers and DARPA will fail to be able to use channels as devices.
- Relevant physical chemistry of ions in channels and solution. These ions are the carriers of electric current that make channels proteins into ionic devices. The special properties of ions and their interface with electrons are key issues in the practical use of ion channels.
- The gating processes that open channels. The opportunities for DARPA are enormous if we learn to control gating. If gating processes can be harnessed, sensitivities to single molecules can be manipulated and exploited. Currents change tens of picoamps in response to binding events of one or two molecules in many ion channels. The opportunity is to build devices using this sensitivity. The challenge is to understand natural biological gating well enough that we can replicate and improve it, manipulating and exploiting it for our use. Present knowledge of gating is too limited to even begin a program in this direction. One way to close this gap is to examine a somewhat speculative ‘flyer’ in which an insight developed from studies of ion movement in already open channels is pursued. Exquisite sensitivity has been noticed in open properties and finds that that sensitivity is characteristic of unstable open states. The mathematics of the open state automatically gives such sensitivity and concomitant gating properties, as a consequence of its instability. The objective is to see if the gating found in the mathematics has properties similar to the gating of real channels.

1.3 - Inter-institutional Relationships

Expertise in the many disciplines addressed above is not found in one institution. Integration of such disparate disciplines requires close intellectual and emotional

interaction to overcome the barriers at boundaries between disciplines. Success depends on previously existing interactions and friendships particularly if it is to be achieved on the DARPA timescale. The work performed under this effort occurred at different institutions strong in each of the required disciplines leveraging established personal and working relationships.

The research and development activity performed as part of this effort was co-coordinated by Dr. Robert Eisenberg, working from Rush Medical Center, and from his company Molecular Biophysics. Rush Medical Center provided the fundamental framework. Molecular Biophysics provided additional time for Dr. Eisenberg to work.

Understanding what needs to be built was the role of Illinois Institute of Technology (IIT) and Rush. Building the experimental device was Arizona State University (ASU). Measuring the device was Rush.

Understanding the Electronics was done at IIT, Rush, and University of Illinois at Urbana-Champaign (UIUC).

Understanding the Experimental Difficulties was done at Rush.

Understanding of the Physical Chemistry was done at UIUC, Rush, and Molecular Biophysics. Cost sharing funds were used to bring in additional expertise from wherever necessary, which may be other continents.

Understanding the biology and technology of proteins was done at Rush and Molecular Biophysics. Rush took the lead in handling the specific properties of ion channels in membranes. Molecular Biophysics took the lead in handling the biochemical properties of ion channels as proteins.

2.0 – Expansion of Theoretical Knowledge of Ion Channels

The initial portion of the research focused on expanding our knowledge of ion channels through simulation and theoretical work. Experiments were performed to see how well porin and its mutants were described by the continuum theories that were being examined and multi-scale simulations that were run. The function of ion channels is a very complex biological process of which theoretical knowledge continues to evolve. A broad range of numerical methods were utilized in the various aspects of this project which have been well documented in a variety of articles. A chapter in the Reviews in Computational Chemistry, Volume 22, brings together the research of not only this team's members but also others interested in ion channels to introduce the numerical techniques required to simulate charge transport in ion channels. [1]

Using Poisson-Nernst-Planck-type (PNP) equations researchers examined how to predict the, "current through an open channel given the spatial structure and fixed charge

distribution." [2] As work progressed it was discovered that there were some limitations to a standard PNP approach and that, "molecular details such as finite size effects and the dielectric force on a discrete ion," must be taken into account. One approach for including molecular details is through a conditional system referred to as C-PNP. [3, 4] With the proper numerical methods the researchers enhanced their theoretical work to explore the selectivity of channels.

Other researchers took on the task of developing an improved force field scheme for the particle-based simulation of ion channel systems. Using a force-field scheme based on a Poisson particle-particle-particle-mesh algorithm, they made, "an initial step toward the development of an efficient and robust simulation tool for the particle-based-modeling of complex inhomogeneous biological systems, such as lipid membranes and ion channels." [5]

Monte Carlo simulations were used to examine ion channel selectivity [6], induced charges in an inhomogeneous dielectric [7], and the equilibrium structure of electrolytes [8]. A three dimensional ion channel simulator was developed, "as an alternative to the computationally intensive Molecular Dynamics approach for simulating ion transport through channels...a promising tool for studying conduction through ionic channels on a timescale relevant to experimental observations." [9]

During this effort considerable progress was made in expanding the theoretical understanding of ion channels. Additional work examining how to properly model ion channels along with understanding their selectivity and sensitivity, that has not been referenced in this section of the report, is documented in the articles listed in the bibliography at the end of the report.

3.0 – Gating

The original intent of the work in this area was to demonstrate selective gating with high signal-to noise ratio (SNR) of porin in response to molecular binding events. This was to be accomplished through the construction of a self-consistent computable model, and the calculation of gating and ionic current transients. Redirection from DARPA to focus more on the development of a silicon based channel, however, prevented this work from being completed. Prior to the redirection, a meeting of leading experimentalists (Dr. Francisco Bezanilla, Dr. Robert Horn), theoreticians (Dr. Zeuss Schuss, Dr. Carl Gardner), and those doing both theory and experiment (Dr. Wolfgang Nonner, Dr. Robert Eisenberg) was held to define the data set and the class of models to be used. Appendix A provides the list of the meetings attendees and the contact information. This meeting lead to the definition of a data set and class of models required to better understand gating.

The illustrations in Figure 1 represent the basic structure on which the modeling could be based.

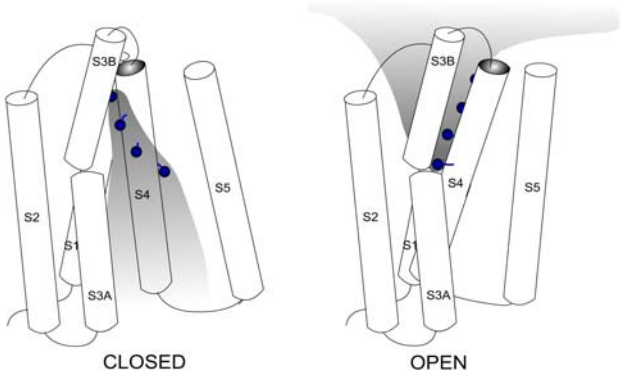


Figure 1. Structure for ion channel gating research.

The key data for the dataset in the gating research is as follows:

1. **Total charge per channel**: Total gating charge moved per channel over the entire range of activation should be approximately 12.5 electronic units. [10, 11]

2. **Rising phase**: Stepping the membrane potential to a potential that is sufficiently depolarizing to cause channel opening produces a rising phase in the gating current. The rising phase is a common feature among voltage-dependent channels. [12]

3. **Fast component**: On the microsecond time scale, gating currents display a fast component that precedes the rising phase and has a time constant of approximately 12 microseconds. [13]

4. **Cooperativity**: Early steps in the activation process demonstrate nearly independent behavior among the four subunits that make up the structure of voltage-sensitive ion channels. The final series of steps leading to the open state, however, demonstrate strong cooperativity among the subunits. The evidence of this is based on kinetic analysis of activation and specific mutations that separate early and late components of gating. A reasonable model of gating must have a mechanism for delaying cooperativity until late into the activation process. [14, 15]

5. **Capacitance of first closed and open states**: The open state is quite inflexible, i.e. narrow and deep. The first state in the activation pathway, on the other hand, appears to be a relatively roomy basin in the gating landscape that spans a total range of about one electronic unit of gating charge per subunit. [16]

6. **Temperature dependence**: The sensitivity of gating to temperature falls into two categories based on the time scale of measurement. On the microsecond time scale, the gating currents have the weak temperature dependence of a diffusion process. On the millisecond time scale, however, the slow gating current that carries most of the gating charge has substantial temperature dependence, indicative of a barrier process. [17, 18]

7. **Cole-Moore shift**. The Cole-Moore shift describes a simple displacement of the ionic current along the time axis with increasingly negative pre-pulse potentials. It is an indicator of multiple closed states along the activation pathway and is seen in most voltage-dependent ion channels. [19, 20]

8. **Nonstationary time course of gating current noise.** Nonstationary variance analysis of gating current noise in sodium and potassium channels demonstrates a time-decaying signal above background, strongly suggestive of "barrier hopping" along the activation pathway, but which could also arise from a spatial dependence of the effective diffusion coefficient that governs the "activation coordinate" of gating. [21, 22]

4.0 - Novel Interfaces to Channels Milestones

This portion of the project investigated important building blocks of a fully integrated biosensor with on-chip sensing and signal processing. The primary goal of this portion of the project was to realize the design of a lipid bilayer support made out of crystalline silicon. The ultimate goal is to create a biosensor based on selective ion channels integrated with detection electronics on a single chip. This would be achieved by realization of the idea that well established silicon microfabrication tools can be used to create a support for a lipid bilayer containing selective ion channels as well as integrating reversible electrodes and electronics on the same chip. A conceptual diagram is shown in Figure 2. Before incorporating the bilayer support into the integrated sensor, a structure was designed that was compatible with the existing patch-clamp measurement setup at Rush Medical in order to provide an easy comparison between their previous transport results and the properties of a lipid bilayer attached to silicon/silicon dioxide.

A preliminary process flow for fabricating the test samples was developed. Because design changes are to be expected, a laser direct-write optical lithography tool was used to pattern the photoresist. This tool has the advantage of significantly reducing the lead-time between changes in design parameters and the actual processing of the wafers. Since the use of thick photoresist is required for the subsequent hole etching step, the tool had to be set up and calibrated to meet the demands for the process.

The first measurements on the oxidized samples performed by Dr. John Tang at Rush Medical Center made it apparent that the parasitic capacitance of the oxidized silicon wafer is too high to allow low-noise measurements on lipid bilayers. Thus, the ASU group developed a method that helps to decrease the capacitance of the setup by using a thick (> 50 μm) photosensitive epoxy layer (SU-8), widely used in microfabrication. Measurements of the silicon chip without and with SU-8 are shown in Figure 3.

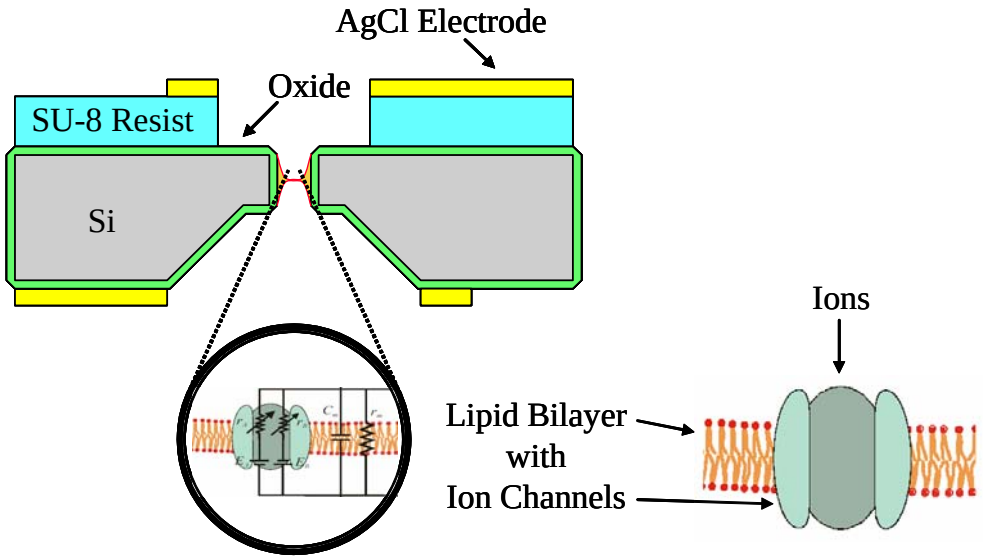


Figure 2. Conceptual illustration of a lipid bilayer with ion channels on silicon

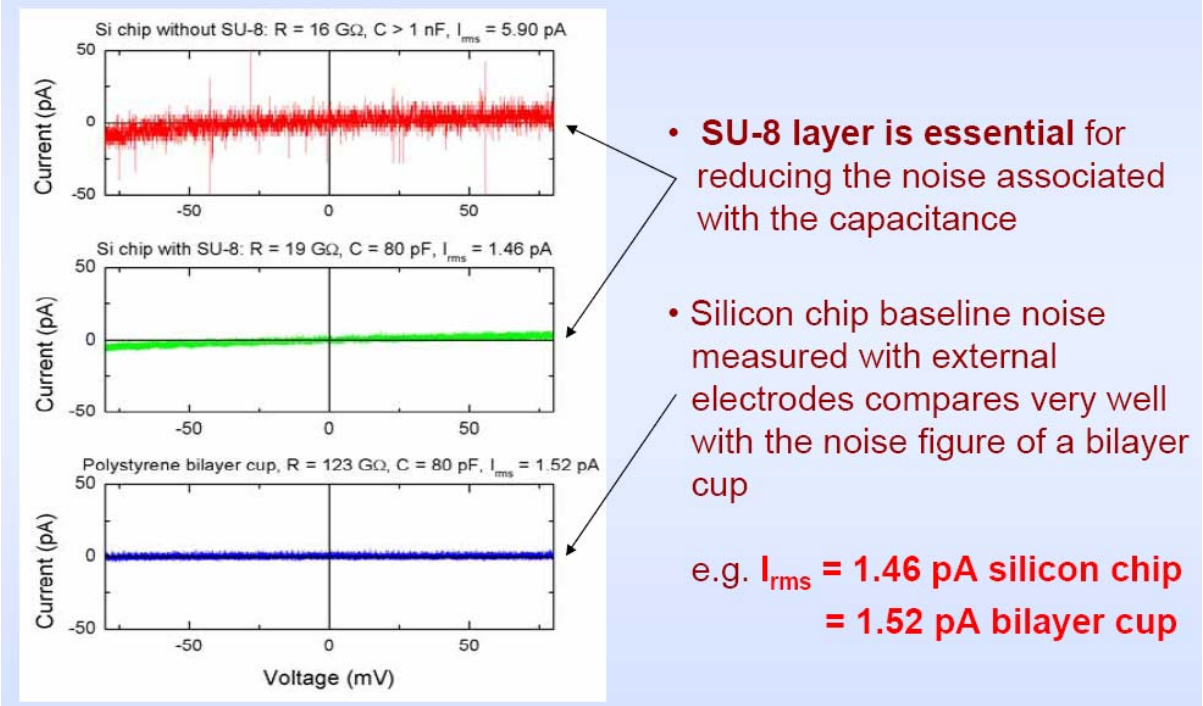
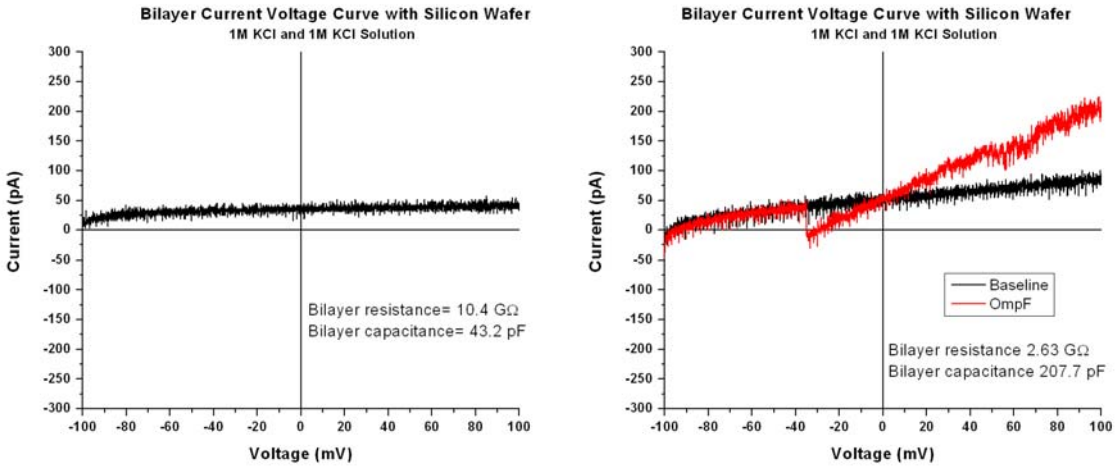


Figure 3. Results of noise measurements showing noise reduction with the use of SU-8 and a baseline for the silicon chip.

After the initial adjustments to the fabrication process, experiments were conducted to examine the formation of the lipid bilayer on the silicon substrate that had an aperture of 250 μm etched through it. Experimental results are shown in Figure 4. In order to

improve the stability of the bilayer the fabrication process was adjusted so the surface supporting the bilayer on the silicon substrate was modified. A plasma chemical vapor deposition (CVD) step was added to deposit a polytetrafluoroethylene (PFTE, Teflon) film on the oxide surface which changes the characteristics of that part of the substrate from hydrophilic to hydrophobic. Researchers have found that the stability of the lipid bilayer is related to the contact angle between the bilayer and the supporting substrate as shown in Figure 5. By adding the Teflon film the contact angle changes from a small to a large angle. Figure 6 illustrates how the contact angle can change based on the modification of a substrate's surface. Those wishing to see more information related to this particular aspect of the project can find them in a Superlattices & Microstructures paper [23] that contains a more in depth discussion of early sample preparation and use of Teflon. An Applied Physics Letters article contains additional discussion on the benefits of the use of Teflon in microfabricating apertures in a silicon substrate and current-voltage measurements taken to demonstrate the robustness of PFTE and reusability of the design.[24].



a.) Determination of the electrical properties of a bilayer that has been formed by painting over the silicon hole using the lipids (DOPE:DOPC, 4:1) dissolved in Hexane. The bilayer could be broken by applying a brief high voltage pulse.

b.) Experiment showing a bilayer with channel activities after OmpF porin insertion. The resistance was derived from the slope of the current trace and the bilayer capacitance was measured using the pClamp software.

Figure 4. Lipid bilayer experiments.

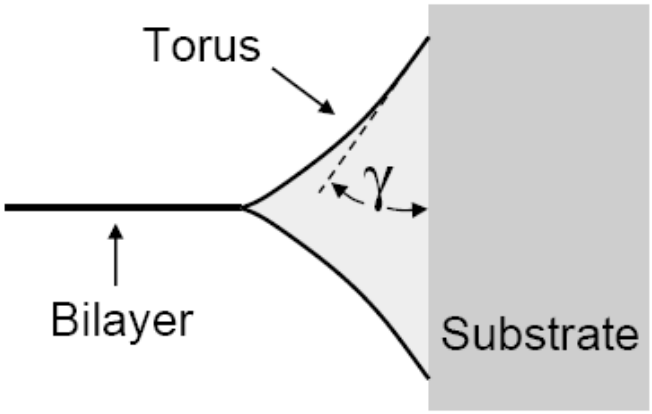
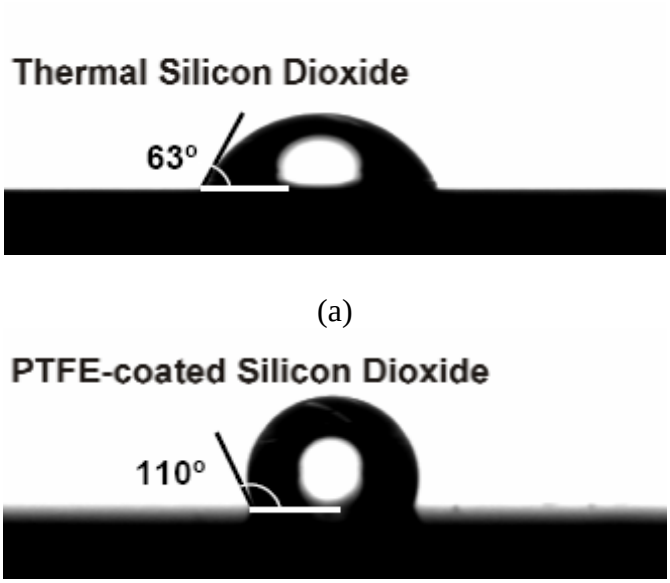


Figure 5. Illustration of the contact angle between the lipd bilayer and the supporting structure.



(a)

(b)

Figure 6. Water droplets on two different surfaces showing how the contact angle changes.

The electrode design was for reversible Ag|AgCl electrodes that were fabricated during a deposition step on the silicon substrate. A mask set that incorporates reversible electrodes on either side of the etched hole was implemented as illustrated in Figures 7 and 8. Figure 9a shows the silver chloride (AgCl) ring on oxide while Figure 9b shows it

on SU-8. Figure 10 shows a close up of the silicon substrate with the electrode and aperture in which the bilayer would be formed.

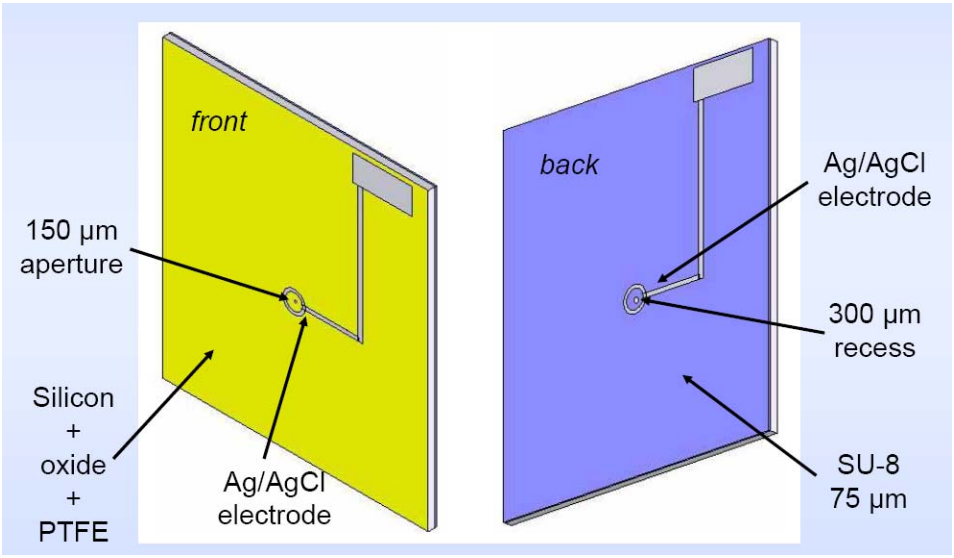


Figure 7. Illustration of design concept showing electrodes and different layers of material.

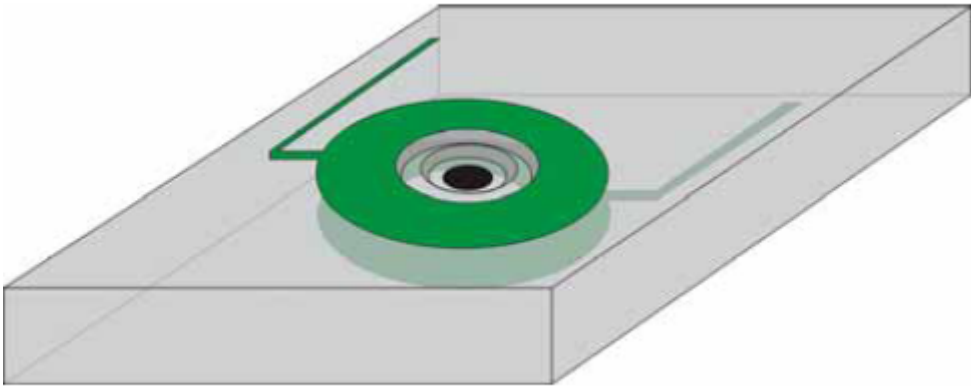
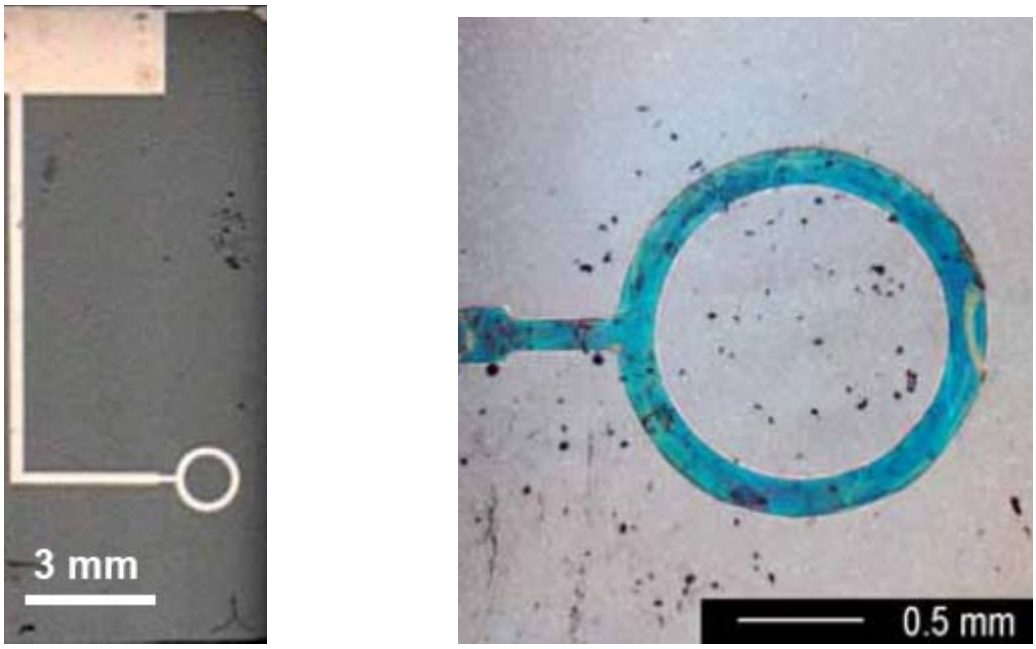


Figure 8. Three dimensional illustration of design concept.



(a) (b)

Figure 9. Images of the circular electrodes that were integrated in to the final design.

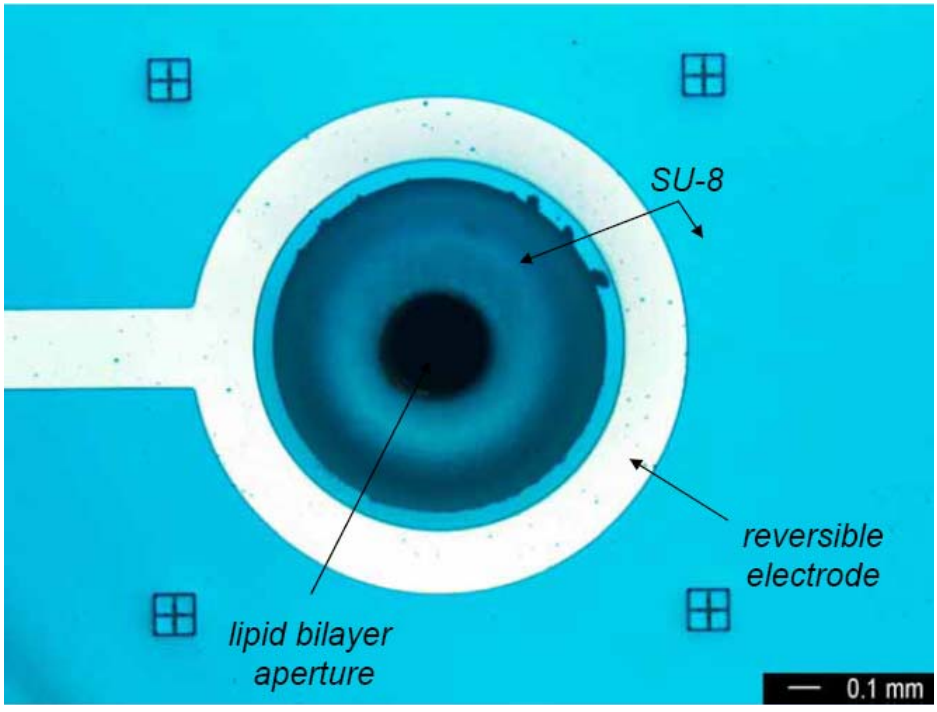


Figure 10. Close up picture of silver electrode on SU-8 epoxy.

Refinement of the microfabrication process was necessary to obtain the desired substrate to demonstrate the fabrication of the supported structure with integrated electrodes. Figure 11 shows the refined process.

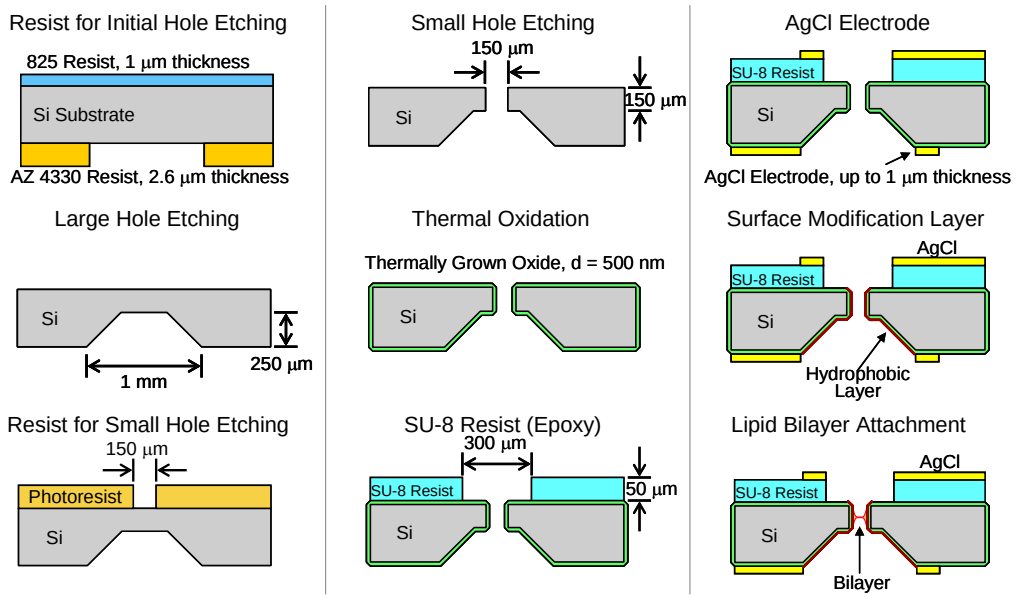


Figure 11. Illustration of process flow.

Testing was conducted on various samples as the design was refined to validate different aspects of the concept for embedding ion channels on silicon evolved. Figures 12 and 13 show the results of measurement made of the electrodes. Figure 14 shows the results of successive bilayer membrane formation attempts. While it is easily seen that the seal resistance drops with successive membrane formation attempts, it should be noted that the initial gigaseal can be restored after cleaning the silicon with distilled water and ethanol. Figure 15 shows the current-voltage traces of outer member protein F (OmpF porin) measured with Ag/AgCl electrodes integrated into silicon device 22 hours after initial bilayer formation and protein insertion. The insets shows single exploded region of the trace with steps corresponding to the single channel conductance of the protein. In addition to the references already mentioned above, additional details explaining the fabrication process and experimentation can be found in articles in the Materials Research Society Symposium Proceedings, Vol. 820 [25] and the e-Journal of Surface Science and Nanotechnology [26].

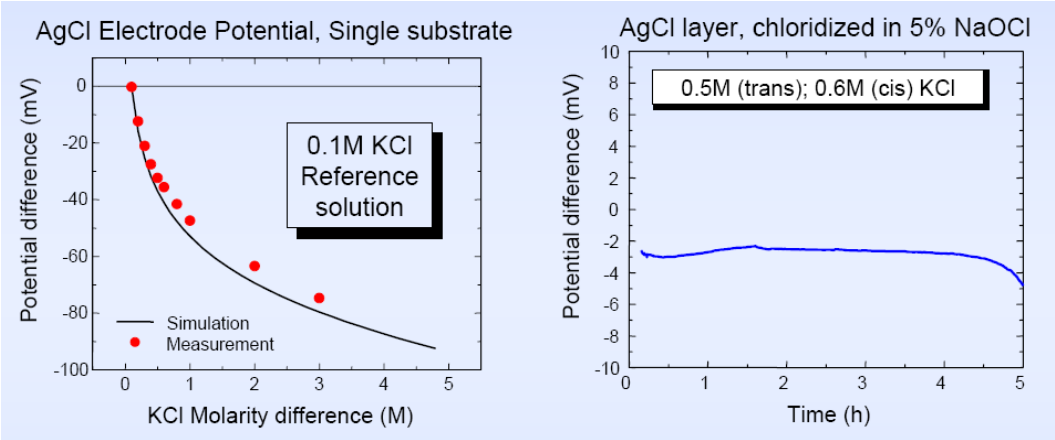


Figure 12. Electrode measurements.

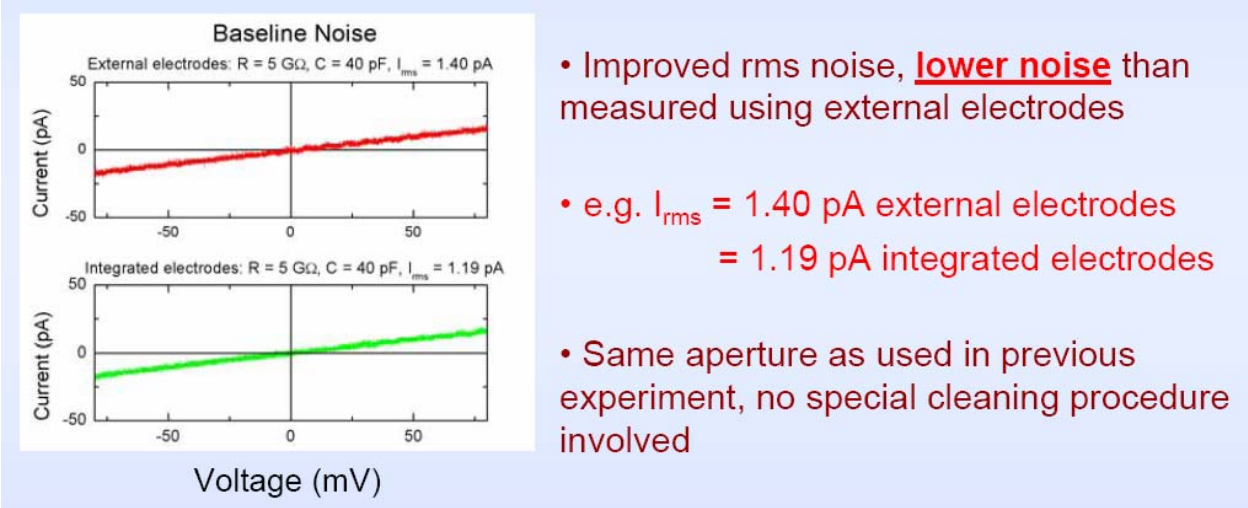


Figure 13. Noise measurement of electrodes.

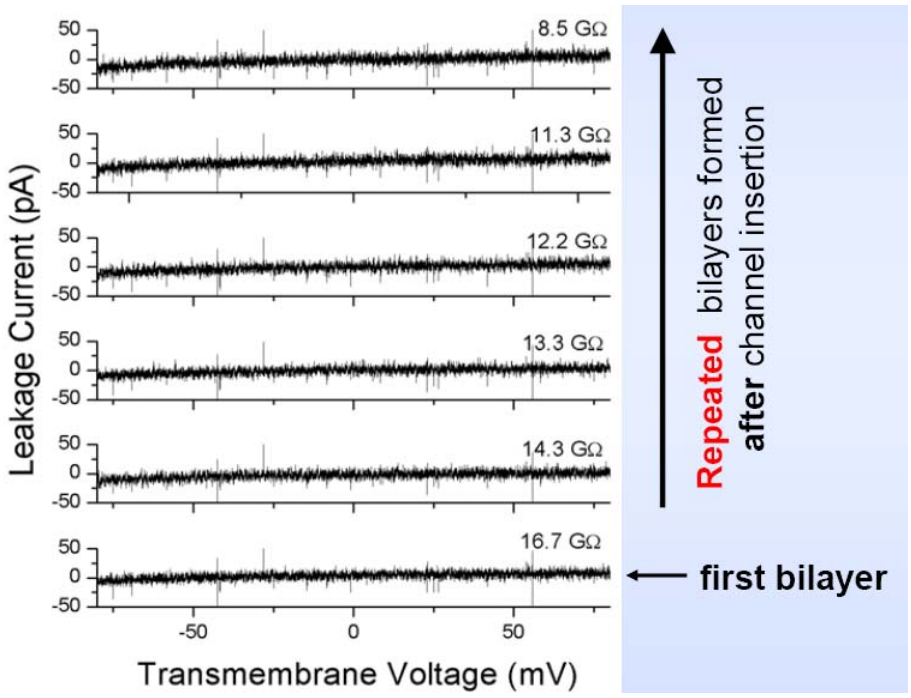


Figure 14. Initial results of forming and reforming lipid bilayers on one device showing the ability to obtain seal resistance each time.

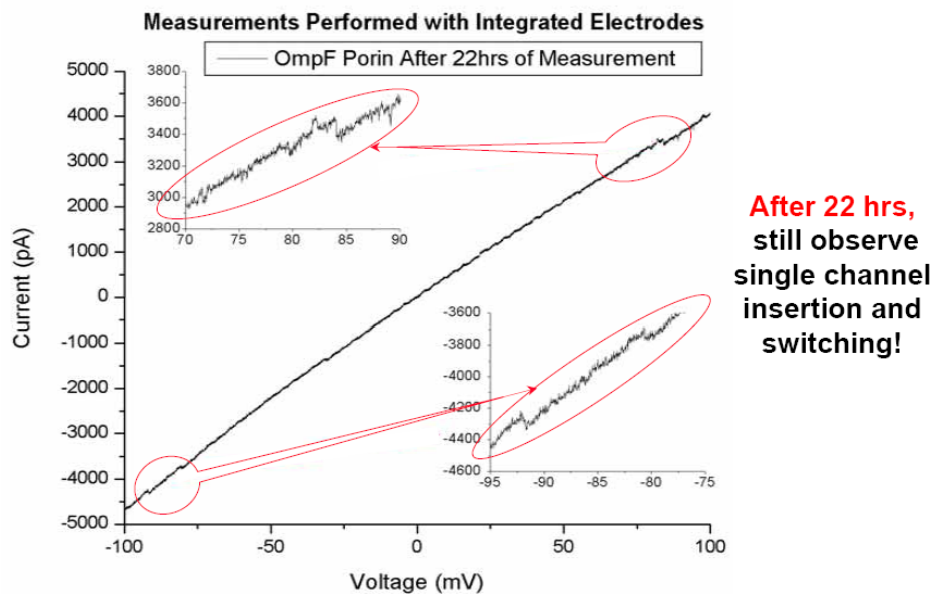


Figure 15. Long term measurements.

5.0 – Concluding Remarks

During this effort progress was made in expanding the theoretical knowledge of ion channels, improving the ability to model ion channels, and demonstrating how one can build a silicon-based ion channel sensor using commercial microfabrication techniques. There is, however, much work that still needs to be done to better understand the selectivity, sensitivity and gating of ion channels in order to develop them for practical applications. As for an integrated sensor, more long term reliability measurements on the order of multiple days and weeks are needed to understand what happens to the bilayer over time. Scalability of the concept must also be examined along with how one would integrate the bilayer chip with microfluidics for automatic bilayer formation and the electronics for automatic measurements and signal processing.

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